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13. ABSTRACT ***(Maximum 200 Words)***
Targeting telomerase RNA (hTR) for degradation by RNAseL in breast cancer cells using antisense molecules linked to 2-5A has demonstrated high levels of cytotoxicity *in vivo* and *in vitro*. The antisense molecules used in preliminary studies carried a phosphodiester backbone which can be recognized by endogenous nucleases and so make the half life of these molecules very short. Having demonstrated *in vitro* that breast cancer cells undergo apoptosis following this treatment, we now sought to improve the stability of the antisense molecules by modifying the backbone. The phosphodiester linkages were therefore replaced with thioate or 2'-O-methyl linkages in various combinations. Many of these modifications resulted in the loss of ability to induce apoptosis in breast cancer cells. Although some of the modified oligonucleotides showed reasonable degrees of cytotoxicity, none of them were as efficient as the original (H1) phophodiester version of the oligonucleotide. When new targets within hTR were challenged with 2-5A-linked molecules, none showed any improvement over H1. We conclude that the H1 antisense molecule has the greatest specificity but that its efficacy is less if the backbone structure is changed either because of a reduced affinity for the target sequence or a reduced ability to activate RnaseL.

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INTRODUCTION

Telomerase is the riboprotein enzyme complex that prevents the ends of chromosomes from shortening below a critical length in cancer cells. This enzyme is normally not expressed in the majority of human cells after an early point in embryonic development but is reactivated in the vast majority (95%) of highly malignant cancer cells. It is thought to be an essential requirement for the maintenance of cell viability in cancer cells which express it. We have investigated whether inactivating telomerase in cancer cells using an antisense oligonucleotide approach targeting the RNA component of the enzyme will result in cell death. The oligonucleotides used carry a 2-5A moiety attached to the antisense molecule. 2-5A activates endogenous RNAseL which is normally found as an inactive monomer in the cytoplasm in most cells. In the presence of 2-5A the monomer dimerizes and becomes a potent RNAse. Thus, the antisense molecule targets a specific RNA and the recruitment of RNAseL then selectively degrades the target. The overall aim of the project, therefore, is to determine whether inactivating telomerase can be developed as a viable form of anti cancer therapy for breast tumors. The initial series of experiments are designed to establish the conditions of treatment which will produce effective cell killing. In the last report we demonstrated that 2-5A-anti-hTR treatment of human breast cancer cell lines resulted in rapid cell death *in vitro* which was due to the induction of apoptosis. When tumors were induced in the flanks of nude mice and treated by direct injection of the antisense oligonucleotides, tumor development was retarded compared with control animals. We had thus demonstrated that growth of human breast cancer cell lines could be prevented by treating with an antisense oligonucleotide targeting the RNA component of telomerase. The main problem with this approach is that, if we are to progress to a more effective treatment *in vivo* then oligonucleotides with a phosphodiester background are unlikely to be stable for more than a few hours; thus, for most of this last year we have focussed on investigating the effects of using modified oligonucleotides in the *in vitro* assay system described in the last report.

BODY

The H1 oligo (Table 1) was designed against the most open part of the predicted structure of the RNA component of hTR. This oligo causes rapid cell death in breast cancer cell lines over 8 days treatment. To investigate whether this indeed was the most effective structure to target, we synthesized another series of oligos from other regions throughout the hTR gene (Table 2) and used these in cytotoxicity assays using the cell line MCF7. The results obtained are shown in F1. A duplicate of H1 was synthesized and coded within this series and the code was only broken after the in vitro studies were completed. Oligo GTI001 was designed from within the promotor region of hTR, and GTI002 was at the initiation of transcription site. It can clearly be seen from the results in Figure 1 that only one of the new oligos (GTI005) performed as well as H1. In fact, when decoded this was the duplicate of H1. A second oligo (GTI004) showed a significant effect over the extended 16-day period but none of the other molecules showed any killing effect at all. From this limited survey we conclude that the original design of the oligonucleotide was still the most efficient. This observation was important firstly to demonstrate that the killing effect is not due simply to oligonucleotide toxicity since, if this were the case, all of the oligos should have had the same effect. Secondly, in a subsequent series of experiments (see below) we began to investigate the effects of modifying the oligos to make them more stable and, because of the high costs of synthesis, we needed to be sure which was the best antisense molecule. The analysis in Figure

2 tested another oligonucleotide, GTI013, which had shown some mild killing effect in human glioma cell lines but, as is shown, is apparently not effective against MCF7.

The original strategy for targeting the open loop using 2-5A linked oligos was that the specific target within the sequence is not important per se but rather that the oligo can bind. Once bound, RNAse L is recruited to the target to degrade the RNA molecule. In a more traditional antisense approach the oligo must bind to a functional region of the template to interfere with transcription or function. The main functional component of the hTR RNA is the template region which is used by the telomerase holoenzyme to preserve the length of human telomeres. To test whether targeting the template region would have any effect on cell viability we constructed an oligonucleotide, GTI116 (Table 1), which carries antisense to the template region. The results of this assay in MCF7 cells is seen in Figure 2. Clearly GTI116 has little effect on cell survival, probably because the template region of hTR is only open over the 6 critical base pair region and so may not allow access of the oligo to the template. Again this result supported our plan to pursue modifications of the original H1 oligo.

The conventional 2-5A antisense oligonucleotides are only protected against rapid degradation by intracellular and extracellular nucleases by the addition of a phosphothioated moiety at the 5' end and a 3'3' inversion of the nucleotide at the 3' end. These modifications make the oligonucleotides resistant to rapid degradation by nucleases but it has been estimated that their activity falls off after approximately six hours *in vitro* (RH Silverman, pers comm). Thus, to develop these drugs for systemic treatment in the future it will be necessary to develop a more stable oligonucleotide. Oligonucleotides can be modified in a number of ways which makes them more resistant to nuclease digestion. One modification is to replace the phosphodiester linkages in the backbone with sulfur linkages. Although complete replacement of the phosphodiester backbone has been one strategy, it is often possible to achieve greater stability by substituting only those nucleotides at the ends of the oligonucleotides. The other modification is to substitute 2'-O-methyl groups for the phosphodiester groups which is a modification seen in tRNA molecules and which makes them naturally resistant to nuclease digestion within the cell. To investigate whether any of these modifications to the 2-5A-anti-hTR affected their ability to inhibit cell growth we created a series of novel oligonucleotides and used them in the *in vitro* assays for several cell lines. A summary of the modified oligonucleotides is given in Table 1.

The first oligonucleotide tested (#48) carries a phosphothioate substitution of the last nucleotides at the 5' end of the H1 oligo. As can be seen in Figure 3, these oligos were effective in killing MDA468 cells over the six days treatment, although these oligos were not as effective as the original unsubstituted anti-hTR oligonucleotide (H1). Oligos substituted with 2'-O-methyl groups throughout the oligonucleotides (86) were much less efficient in killing MDA468 cells (figure3). From these experiments it was shown that the original H1 oligonucleotide was still the most efficient for killing cells *in vitro*. The issue was, however, whether the oligos would be more stable; to investigate this, we treated cells with the various test oligos at 48-hour intervals rather than 24-hour intervals. The results are shown in Figure 4. These results demonstrate again that the phosphothioate substituted oligo (102) has no effect on cell growth in either treatment. The same was true for oligonucleotides where the 2'-O-methyl nucletide residues were used with (106) or without (104) phosphothioate substitutions as well. The oligo (108) with 2'-O-methyl substitutions at all of the last three nucleotides, however, appeared to have a greater killing effect but again not as efficient as the original H1 oligo. When the cells were treated every 72 hours (Figure 5), there is no effect of any of the oligos suggesting that the population of cells after an initial response can then recover indicating that none of the oligos can persist for the 72-hour period. In summary

therefore, these results show that modifications of the oligos either abolished their effectiveness or gave no appreciable advantage over the original H1 oligonucleotide. These small-scale preparations of very specific oligos involved a very lengthy preparative procedure and were also very expensive to produce. Given that they showed no appreciable increase in effectiveness we decided not to continue to test further modifications especially since the H1 molecule was so efficient and we have obtained industrial quanities of this reagent.

The original experiments clearly showed that the response of the cells to H1 treatment was apoptosis and so active cell death was occurring in response to targeting hTR. To investigate this effect further we treated cells with the H1 oligonucleotide in the presence of caspase inhibitors. Indeed, in these experiments (data not shown) the cytotoxicity was reduced by up to 40% in the cells treated with inhibitors demonstrating that apoptosis is the mechanism by which cells are killed. The BCL-2 gene is also an inhibitor of the apoptotic pathway and so we tested whether induction of high levels of BCL2 could prevent the cellular response to anti-hTR treatment. An HeLa cell line (kind gift of Dr. A. Almasan), which contains a tet-inducible BCL2 gene, was treated with the H1 and control oligonucleotides and the cell viability was measured in the presence of tetracycline. In these experiments HeLa cells were as responsive to anti-hTR treatment in the presence of tetracycline (and so increased levels of BCL2) as they were in the absence of tet (Figure 6). These experiments suggest that BCL2 is not involved in the cellular response to anti-HTR. To establish whether any significant difference in response could be seen with reduced concentrations of oligonucleotides, we treated HeLA cells in the presence of tetracycline with varying concentrations of 2-5A anti-hTR. As can be seen in Figure 6, the cell killing effect was reduced according to the concentration of oligonucleotides used. This was the same response that was seen in the absence of tetracycline and consistent with our earlier studies which show that 0.5 μM is the effective killing dose of 2-5A antisense.

Through this very extensive series of experiments we have demonstrated further the specificity of the H1 oligonucleotide in causing apoptosis in breast cancer cell lines. None of the other targets with hTR were as effective. Modification of the oligos to make them more stable had the unexpected consequence of reducing or ablating the efficiency of the H1and other oligos to cause apoptosis in breast cancer cells. We are still unclear about the exact mechanism of induction of apoptosis in breast cancer cells but it is clearly not due to decreasing the expression of BCL2.

KEY ACCOMPLISHMENTS

- Demonstration that the target predicted by the structure of hTR is the only one which is effective in the cytotoxicity response in breast cancer cell lines.
- Established that modifications of the antisense molecules using thioate linkages are not as effective in cytotoxicity as the original phosphodiester backbone structure of the anti-hTR oligonucleotide.
- Established that 2'-O-methyl modification of the oligonucleotides compromises the efficiency of the cytotoxicity effect of anti-hTR
- Clearly altering the structure of the antisense molecule interferes with its ability either to recognize and bind to the target sequence or its ability to recruit RNAse L.
- Treatment of breast cancer cells with the H1 oligonucleotide every 24 hours is just as efficient as treating every 24 hours.
- The induction of apoptosis in breast cancer cell lines is not dependent on the expression levels of BCL2.

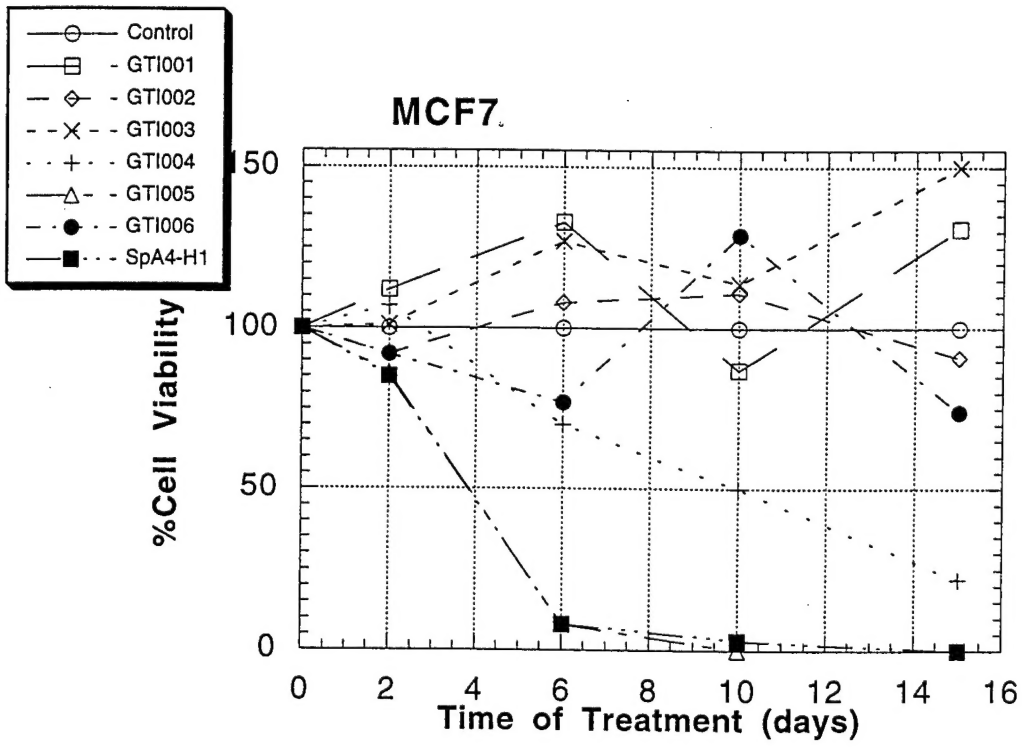
REPORTABLE OUTCOMES

None

CONCLUSIONS

We have investigated the effect of modifying the backbone structure of the oligonucleotides used in our antisense strategy directed against the RNA component of telomerase in breast cancer cell lines. This series of experiments was initiated because of the well-known susceptibility of phophodiester linkages in these molecules to the degradation effects of endogenous nucleases. If any systemic treatment of breast cancer is to be developed using anti-sense strategies, then it is important to stabilize the molecule against degradation. Despite a protracted series of experiments designed to investigate the efficacy of substitutions with sulfur and 2'O-methyl linkages in the H1 oligonucleotide we, unfortunately demonstrated that these modifications apparently interfered with the cytotoxicity capacity of the original oligonucleotde. This was unexpected since these types of stabilization have proved effective in other antisense experiments. In our case, however, we are relying not only on the binding of an oligo to a very closed structure but also the requirement of the dimerization of the Rnase L molecule for the antisense to be effective. It is possible, therefore, that either of these two requirements are compromised by the modifications. We were able to show that several other regions of the hTR molecule were ineffective in causing apoptosis in breast cancer cells, reinforcing the observation that this H1 target is the most efficient. We were also able to show that extending the interval between treatments led to no reduction in cytotoxicity. Finally, in preliminary experiments designed to look at the mechanism of induction of apoptosis, the BCL2 gene, which normally protects tumor cells from apoptosis, does not have a significant role in the cytotoxic effect.

Figure 1: Cell survival curves for MCF7 cells treated every 24 hours with the H1 oligonucleotide. The only effective oligo was GTI005 which when decoded was the same as H1. All of the other oligos either showed no appreciable effect, or a much lower (GTI004) effect than H1.



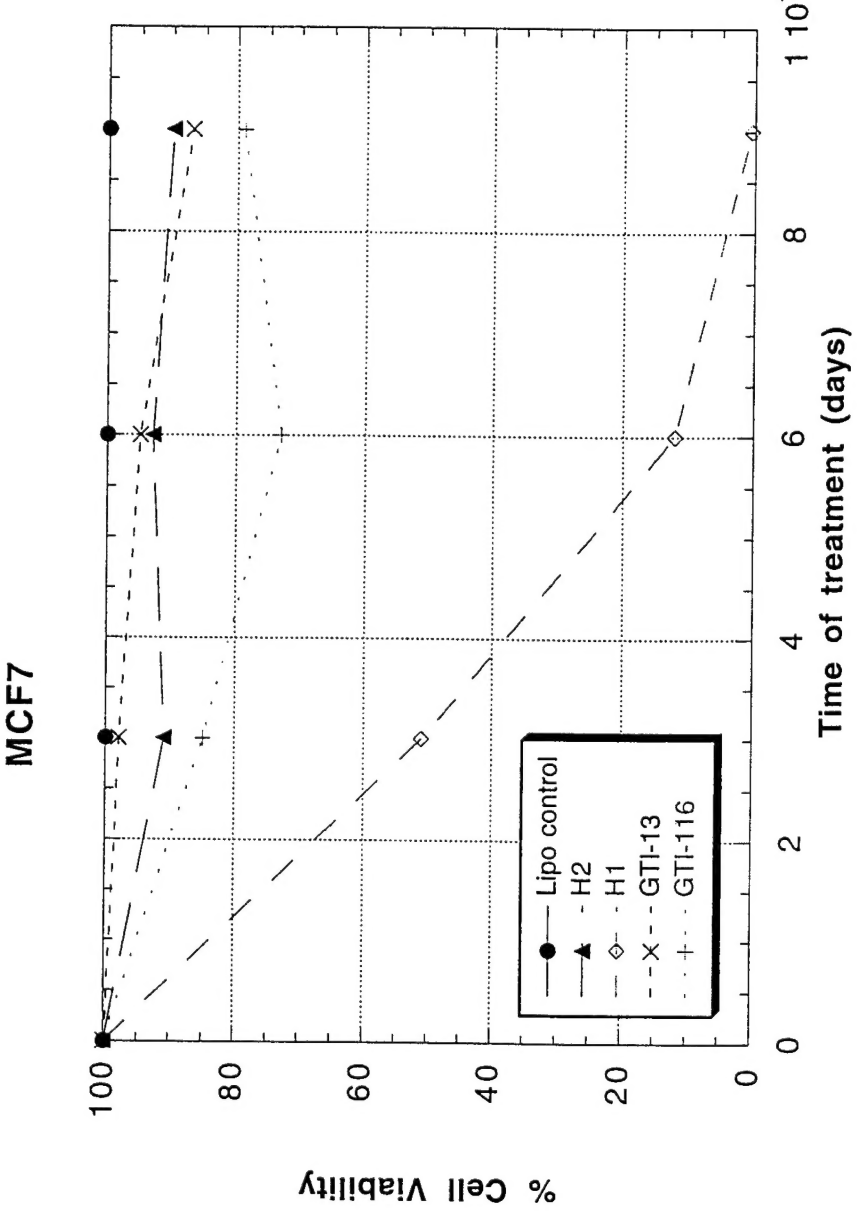


Figure 2. Cell viability assays for GTI013 and the GTI116 oligo which targets the template region of hTR. As shown neither of these olgios have any effect compared with H1.

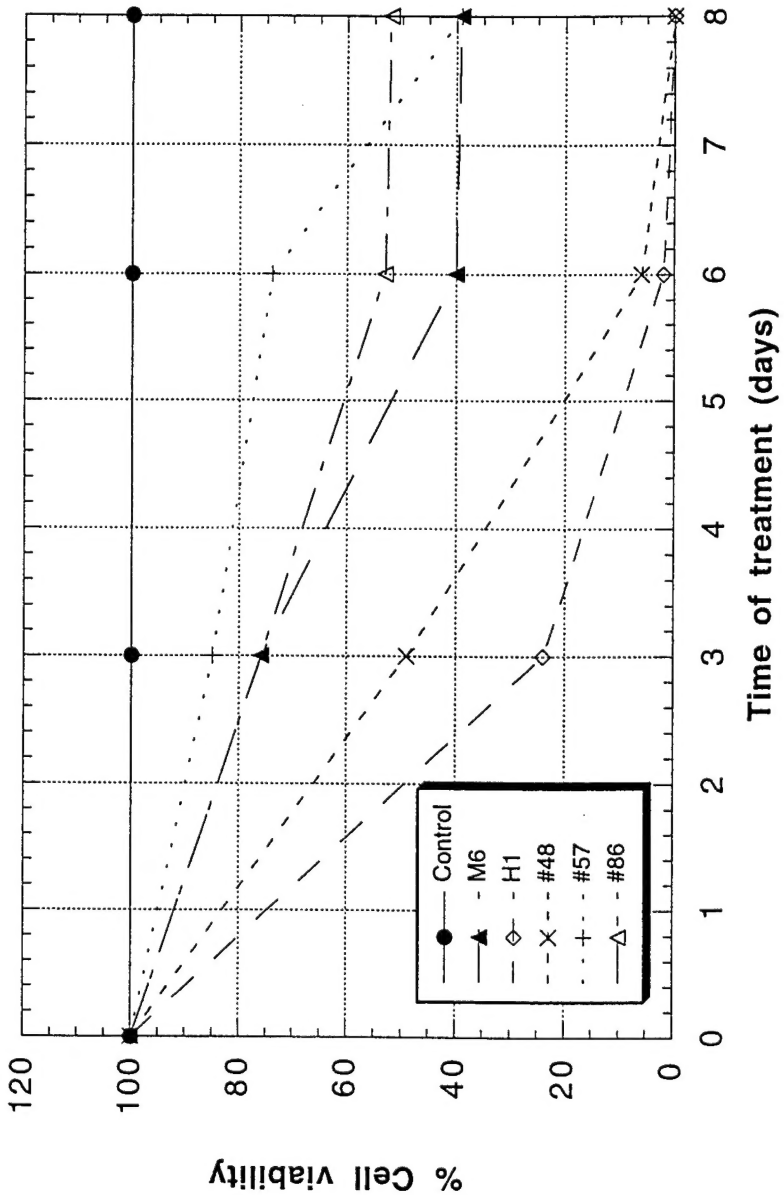


Figure 3.The effect of modified oligos on cell viability in MDA468 cells. Oligos 57 and 86 with 2'-O-methyl substitutions show a reduced effectiveness whereas oligo 48 with a phophothioate substitution at the 5' end shows a similar but weaker cytotoxicity compared with H1.

Figure 4. Cytotoxicity assays for the H1 oligonucleotde with 2'-O-methyl and thosphothioate substitutions (table 1). Treatment with H1 and 108 (2'-O-methyl at the 3' nuclotides only) show a similar cytotoxicity. All of the other substitutions had variable but significantly lesser effects than H1.

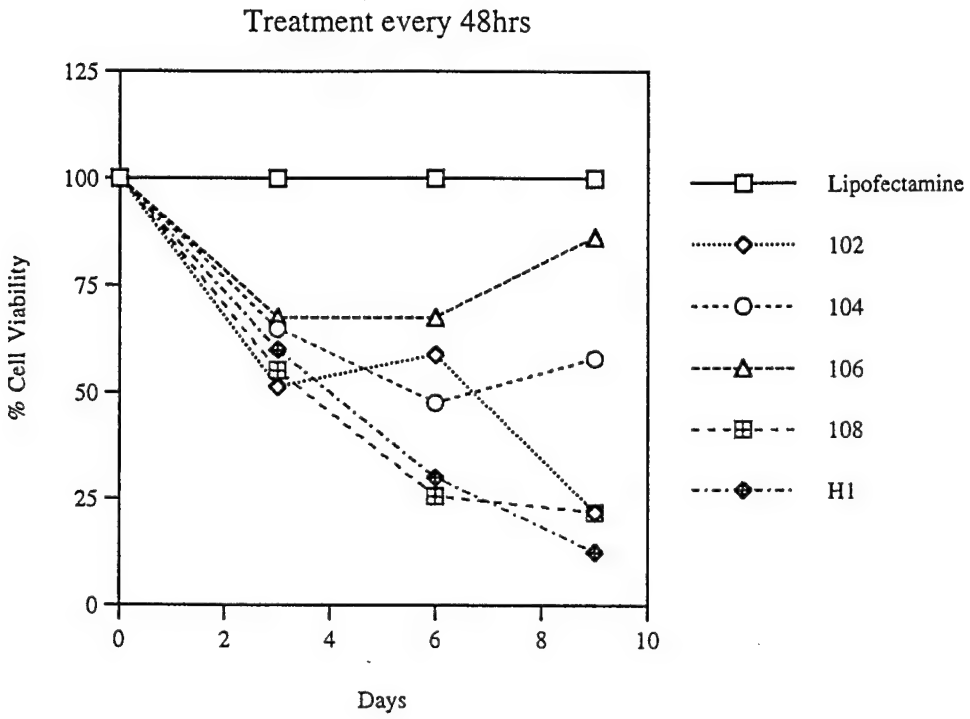
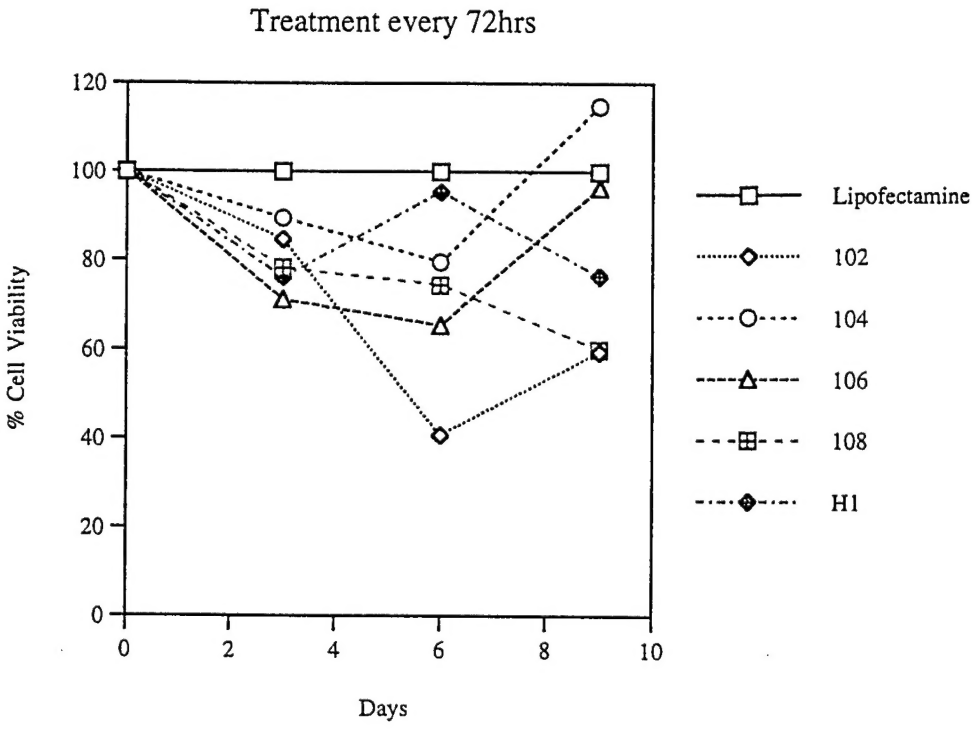


Figure 5. Treating cells with any of the modified oligonucleotdes as well as H1 every 3 days could not reproduce the cytotoxicity seen after 24 or 48 hour treatment intervals.



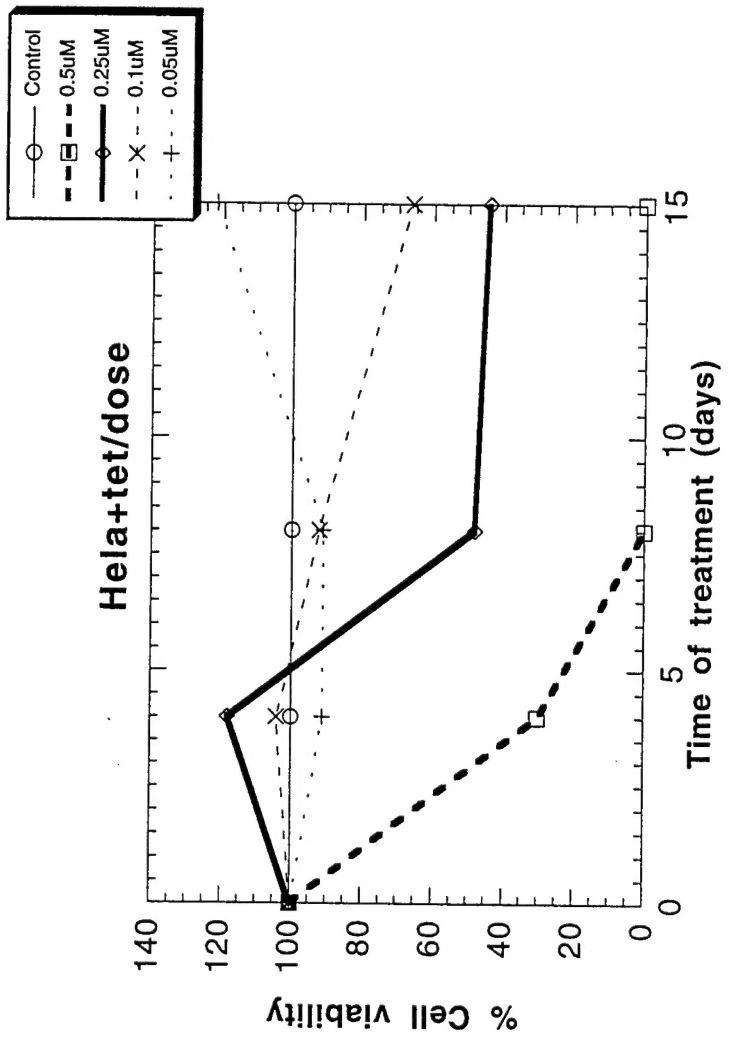


Figure 6 HeLa cells carrying a tetracycline-inducible BCL2 gene were treated with H1 in the presence of tetracycline and so elevated levels of BCL2. Using the standard 0.5 uM dose the presence of high levels of BCL2 could not protect the cells from apoptosis. Lower doses of the H1 oligo simply reduced the cytotoxicity which is also the case in the absence of high levels of BCL-2.

Table 1: Sequences for oligonuclotides and modifications used in cytotoxicity experiments. S = phosphothioate substitutions. M = 2'-O-methyl substitutions.

H1	spA4-d(GCGCGGGGAGGGGGCAAAAGCA-3'-3'T5')
102	spA4-d(GCGCGGGGAGGGGGCAAAAGsCsAs-3'-3'T5')
104	spA4-d(GCGCGGGGAGGGGGCAAAAGCA-3'-3'T5')2'O-Me
106	spA4-d(GCGCGGGGAGGGGGCAAAAGsCsAs-3'-3'T5')2'O-Me
108	spA4-d(GCGCGGGGAGGGGGCAAAAGmCmAm-3'-3'T5')
GTI013	spA4-d(AGTGGGTGCCTCCGGAGA3'-3'5)
GTI116	spA4-d(CAGuuAGGGUUAG3'-3"T5')2'O-Me

gagtgactct cacgagagcc gcgagagtca gcttggccaa tccgtgcggt cggcggccgc
GTI001
tccctttata agccgactcg cccggcagcg caccgggttg cggagggtgg gcctgggagg
GTI002
ggtggtggcc atttttgtc taaccctaac tgagaagggc gtaggcgccg tgcttttgct
H1
ccccgcgcgc tgtttttctc gctgactttc agcgggcgga aaagcctcgg cctgccgcct
GTI003
tccaccgttc attctagagc aaacaaaaaa tgtcagctgc tggcccgttc gcccctcccg

gggacctgcg gcgggtcgcc tgcccagccc ccgaaccccg cctggaggcc gcggtcggcc

cggggcttct ccggaggcac ccactgccac cgcgaagagt tgggctctgt cagccgcggg
GTI004
tctctcgggg gcgagggcga ggttcaggcc tttcaggccg caggaagagg aacggagcga

gtccccgcgc gcggcgcgat tccctgagct gtgggacgtg cacccaggac tcggctcaca

catgc

Table 2. DNA sequence of the hTR gene. The location of the primers used in in vitro assays are underlined